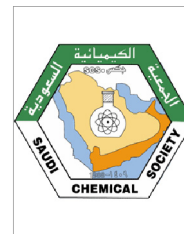




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ORIGINAL ARTICLE

Development and validation of a UPLC method for quantification of antiviral agent, Acyclovir in lipid-based formulations



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KEYWORDS

Acyclovir;
UPLC;
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Validation

Abstract *Purpose:* The objective of the current study is to evaluate the Ultra Performance Liquid Chromatography (UPLC) method for quantification of Acyclovir in lipid-based formulations.

Method: A simple, rapid, reliable and precise reversed phase UPLC method has been developed and validated according to the regulatory guidelines, which composed of isocratic mobile phase; 0.25% formic acid (FA) in Milli-Q water with a flow rate of 0.5 ml/min, and column BEH C18 (2.1 × 50 mm, 1.7 μm). The detection was carried out at 254 nm.

Results: The developed UPLC method was found to be rapid (1.2 min run time), selective with well resolved Acyclovir peak (0.89 min) from different lipid matrices and sensitive (Limit of Detection (LOD) was 0.3 ppm and Lower Limit of Quantification (LLOQ) was 1 ppm). The accuracy and precision were determined and were perfectly matching with the standard FDA limits.

Conclusion: The study showed that the proposed UPLC method can be used for the assessment of drug purity, stability, solubility and lipid-formulation release profile with no interference of excipients or related substances of active pharmaceutical ingredient.

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1. Introduction

Acyclovir (ACV) is chemically known as 2-amino-1,9-dihydro-9-[(2-hydroxyethoxy)methyl]-6H-purin-6-one (Fig. 1). Acyclovir is the most commonly used oral antiviral agent for the treatment and prophylaxis of initial and recurrent episodes of genital and labial herpes and also for the acute treatment of herpes zoster and the varicella (chickenpox) in immunocompetent individuals. As an antiviral therapy it is extremely selective and low in cytotoxicity. According to the pharmacokinetics data ACV is poorly water soluble and it has low, variable and incomplete absorption which leads to

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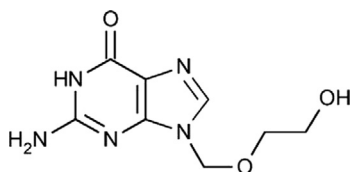


Figure 1 Chemical structure of Acyclovir (MW: 225.21, pKa: 2.27 and 9.25).

poor bioavailability ranging from 10 to 30%. Maximum plasma concentration is reached within 1.5–2.5 h, when ACV is administered orally (Arnal et al., 2008).

ACV was previously analyzed with several analytical methods like High Performance Liquid Chromatography (HPLC) (Teshima et al., 2003, Basavaiah et al., 2003), micellar liquid chromatography (Peh and Yuen, 1997; Macka et al., 1993), gas chromatography (Leis et al., 2002, Rakestraw, 1993), capillary electrophoresis (Vo et al., 2002, Reichova et al., 2002), radioimmunoassay (Tadepalli and Quinn, 1996), and potentiometry (Abdel-Ghani et al., 2002). None of these methods were specially developed in lipid based formulations except biological fluids and few in Acyclovir dosage forms (suspension). There are many drawbacks with the previously mentioned analytical methods such as lengthy and tedious process, lack of required sensitivity or may have required synthetic/special reagents or detectors. Thus, an improved analytical method for ACV should be developed demanding more sensitivity, speed and also required to meet the stability-indicating parameter.

In the current project, the assay method for ACV was developed and validated using a relatively new Ultra Performance Liquid Chromatography (UPLC) system that carries many advantages over HPLC or any other conventional analysis method which reduces analysis time and solvent use significantly. The solvent used as the mobile phase was only water mixed with 0.25% formic acid. Within the experimental method development, it was also needed to carry out stability studies under forced acidic, alkaline, thermal, and oxidative degradation processes. The special feature of UPLC device allows for the system to withstand high back pressure without any harmful effect to the analytical column or the whole device (Abdel-Hamid et al., 2012, Nováková et al., 2006). This proposed method was also successfully applied to the analysis of lipid-based formulations containing ACV with no interference from dosage form excipients. The method was validated with respect to the standard FDA guidelines (US Dept. of Health and Human Services, 2001).

2. Materials

ACV (purity >99.5) was generously donated by Riyadh Pharma Medical and Cosmetic Products Co. LTD, Riyadh 11451, Saudi Arabia. Imwitor 308 (I308) was kindly supplied by Sasol Germany GmbH (Werk Witten, Witten-Germany). Propylene glycol (PG) and Tween 80 (T80) were obtained from Winlab, BDH and Merck-Schuchardt. The mobile phase was HPLC-grade 0.25% formic acid which was prepared by adding 5 ml of formic acid (obtained from BDH laboratory, BDH Chemicals Ltd., Poole, UK with purity of 98–100%) to two liters of Milli-Q water. The high purity Milli-Q water was obtained through a Milli-Q Integral Water Purification System (Millipore, Bedford, MA). All other reagents were of analytical grade and used without further purification.

3. Methods

3.1. UPLC chromatographic conditions

Chromatographic separation was developed and optimized with respect to the stationary and mobile phase compositions, flow-rate, sample volume, and detection wavelength. The study employed a highly sensitive UPLC system that consisted of an Acquity® UPLC binary solvent manager equipped with an Acquity® automatic sample manager and a Photodiode Array (PDA) eλ detector obtained from Waters (Waters Inc., Bedford, MA, USA). Separation was achieved by reverse-phase isocratic elution using a mobile phase consisting of 0.25% formic acid (0.25% FA) in Milli-Q water delivered at a flow rate of 0.5 ml/min through an Acquity® UPLC BEH C₁₈ column (2.1 × 50 mm, 1.7 μm) kept at 50 °C. The run time was 1.2 min. Freshly prepared mobile phase was filtered through an online 0.20 μm filter and degassed continuously by an online degasser within the UPLC system. The detector wavelength was set at 254 nm and the injection volume was 1.0 μl.

3.2. Preparation of stock solution, calibration standards and QC samples

Stock solution of ACV was prepared by dissolving 50 mg ACV powder in 50 ml distilled water, resulting in a solution containing 1000 ppm ACV. Serial standard dilutions (9 points) of ACV were prepared to cover the concentration range of 1–400 ppm. These standard solutions were stored at 4 °C before use. Calibration curves were obtained by plotting peak area against standard drug concentration and regression equations were computed thereby. Four quality control (QC) samples with the selected concentrations (1.48, 64.48, 287.76, and 377.12 ppm) were prepared to cover the desired range. QC samples were prepared by spiking the lipid-formulation with known amount of ACV, then diluting the mixture with appropriate volume of water containing 0.25% FA.

3.3. Formulation matrix effect

Direct spectrophotometric analysis of the drug compound may experience spectral overlapping between the analyte and excipients used in the dosage form, which significantly affects the sensitivity, accuracy and precision of the method (Yu et al., 2008). Therefore it was required to perform UPLC spectral scanning for both drug-containing and drug-free formulations to detect any possible interference. In this study, the analyte (ACV) was loaded in lipid based formulation from low to high concentrations in order to test the extent of the matrix effect..

3.4. Bioanalytical method validation

The above mentioned developed method was validated in terms of linearity, specificity, precision and accuracy, Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ) according to the standard guidelines of bioanalytical method validation (US Dept. of Health and Human Services, 2001; Gao et al., 2011) by FDA.

3.4.1. Linearity and range

Appropriate volumes of ACV stock solution (1000 ppm) were utilized to prepare nine non-zero standard drug concentrations covering the calibration range of 1–400 ppm. Four different QC samples were prepared by spiking known concentrations of ACV within the detection range of 1–400 ppm.

Each standard solution (1, 2, 5, 10, 20, 50, 100, 200 and 400 ppm) has been injected six consecutive times daily on three consecutive days for validation. Calibration solutions were injected in ascending order in each validation run and the other samples were distributed randomly through the run.

Linear regression equation and correlation coefficient (R^2) were employed to statistically evaluate the linearity of the results (Ali et al., 2006; Lister, 2005; Al-Hadiya et al., 2010).

3.4.2. Specificity

Specificity of the method needed to be assessed for the evaluation of the matrix effect between the drug and different lipid-based formulations (details in matrix effect section).

To evaluate the specificity of the method, drug free lipid formulation (QC_{zero}) samples were carried out through the assay procedure and the retention times of the lipid formu-

lation components were compared with those of ACV analyte (Al-Hadiya et al., 2010). In addition, specificity of the method toward the intact drug was also studied by determination of the resolution (R) between the drug peak and the nearest degradation product if present (Dongre et al., 2008).

3.4.3. Accuracy and precision

Intra-day accuracy and precision evaluations were performed using six replicate determinations of nine ACV standards within the same day. Inter-day accuracy and precision were assessed by six replicate analyses of the following: LLOQ, low, medium and high QC samples on three consecutive days. The overall precision of the method was expressed as relative standard deviation and accuracy of the method was expressed in terms of % drug recovered (Gao et al., 2011).

3.4.4. Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ)

The LOD and LLOQ were determined by serial dilutions of ACV stock solutions in order to obtain signal to noise (S/N) ratio of at least $\approx 3:1$ for LOD and $\approx 10:1$ for LLOQ (Lister, 2005).

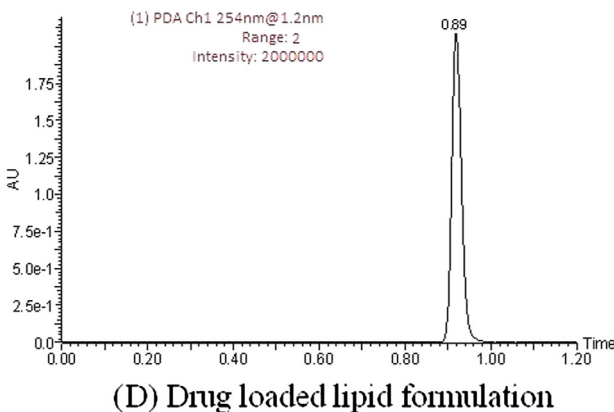
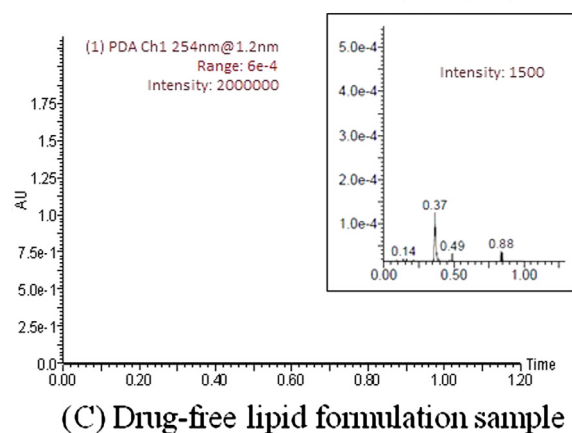
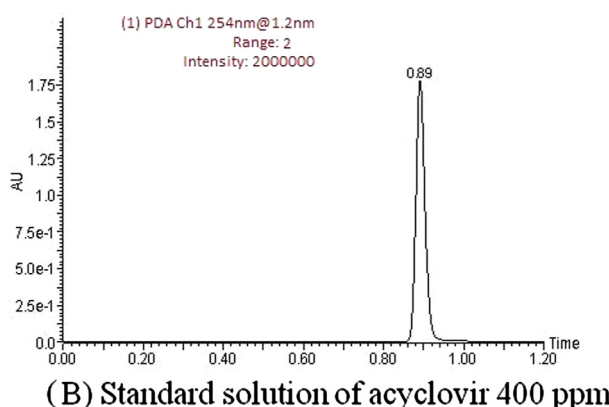
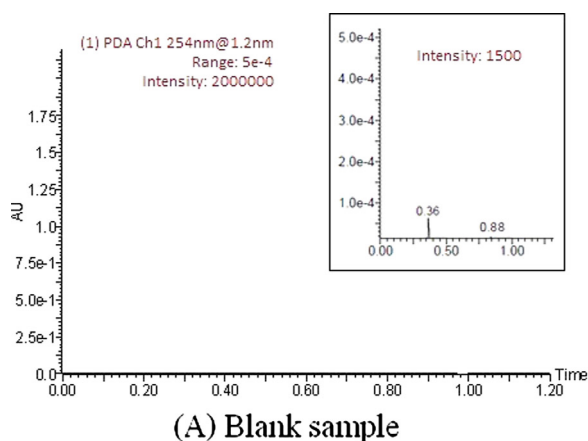


Figure 2 UPLC chromatograms of blank sample (A), standard solution of ACV at concentration of 400 ppm (B), drug-free lipid formulation (QC_{zero}) sample (C), and drug-containing lipid formulation (QC₂) sample (D).

4. Results and discussion

4.1. Chromatographic separation

Fig. 2 shows the representative chromatograms of blank sample (A), standard solution of ACV (B), drug-free lipid formulation (QC_{zero}) sample (C), drug-containing lipid formulation (QC₂) sample (D).

Separation and detection of ACV without any interference were ideal by the developed UPLC assay. The chromatographic result of UPLC technique in the current study shows that the sensitivity and selectivity of this procedure are good enough to determine ACV within the available excipients. The ACV analyte was well separated at a retention time of

~0.89 min without any interference from the excipients (Fig. 2A–D). The total run time was ~1.2 min, where the peaks were of good shape and completely resolved.

4.2. Matrix effect

The assessments of matrix effect represented an integral part of validation for quantitative analysis of drug in lipid based formulations within this analytical method development. The matrix effects at four concentration levels of ACV were assessed. In the present analysis, the overall process efficiency demonstrated that the proposed method was practically free from relative matrix effects for the determination of ACV in lipid based formulation.

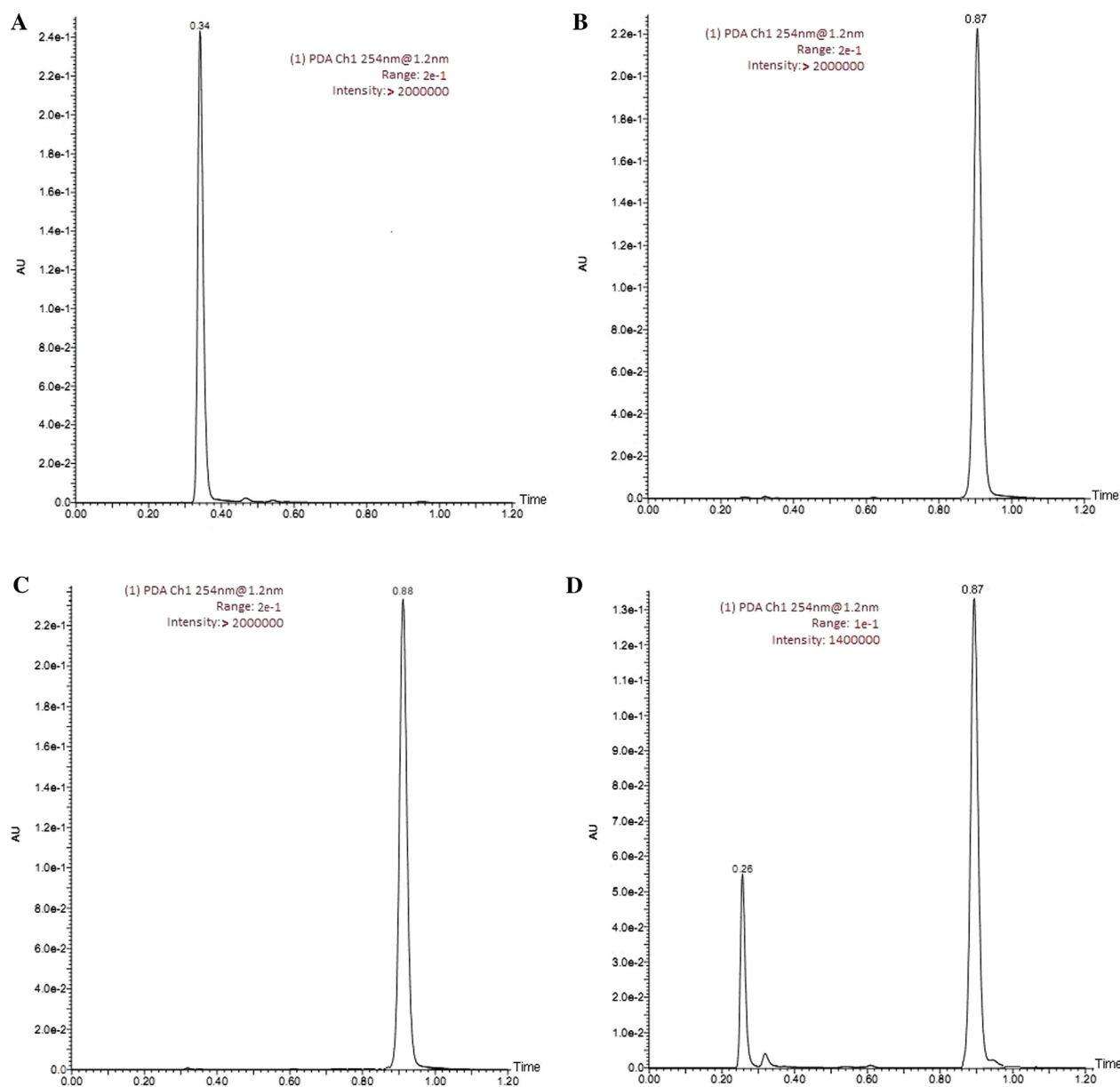


Figure 3 Typical UPLC chromatograms of: (A) acid hydrolysis-degraded ACV, (B) base hydrolysis-degraded ACV, (C) oxidative-degraded ACV, and (D) thermal-degraded ACV.

4.3. Forced degradation study

The force degradation experiment in the studies was conducted by treating model drug ACV with 1 N HCl, 1 N NaOH, 3% H₂O₂ under 100 °C temperature for 2 h at water bath. In case of thermal degradation the experiment was carried out under 120 °C temperature in an oven. All the ACV sample solutions used in forced degradation studies were prepared with final concentrations of 400 µg/ml (400 ppm). The results of the degradation study showed that the concentrations of ACV in the samples were lowered compared to the original concentrations. This degradation study was particularly important for the analysis of lipid based formulation, which was not performed previously (Basavaiah et al., 2003, Macka et al., 1993).

4.3.1. Acid degradation

This study has been made to show the degradation products of ACV using the acid degradation method. ACV stock solution of 400 ppm was prepared; 10 ml of it was transferred to a 100 ml volumetric flask and then 10 ml of 1 M hydrochloric acid solution was added. The solutions in the flask were mixed well and kept for 2 h at 100 °C temperature. After cooling the solution at room temperature, 10 ml of 1 M sodium hydroxide solution was added to a 100 ml volumetric flask to neutralize and then diluted to 100 ml with the mobile phase, 0.25% FA in water. The chromatogram of the acid degradation is shown in Fig. 3A.

4.3.2. Basic degradation

10 ml of the stock solution (400 ppm) was transferred into a 100 ml volumetric flask and treated with 10 ml of 1 M sodium hydroxide solution. The contents of the flask were mixed well and kept for 2 h at 100 °C temperature. After cooling the solution at room temperature, 10 ml of 1 M hydrochloric acid solution was added to a 100 ml volumetric flask to neutralize and then diluted to 100 ml with the mobile phase, 0.25% FA in water. The result of the basic degradation is shown in Fig. 3B.

4.3.3. Oxidation

10 ml of the stock solution (400 ppm) was transferred to a 100 ml volumetric flask and treated with 10 ml of 3% hydrogen peroxide H₂O₂ and then heated in water bath at 100 °C for 2 h. The sample was cooled to room temperature and then the volume completed to 100 ml with the mobile phase 0.25% FA in water. The sample was injected in the UPLC system. The chromatogram of the oxidation data is shown in Fig. 3C.

4.3.4. Thermal degradation

Thermal degradation experiment was conducted at elevated temperature. 10 ml of the stock solution (400 ppm) was transferred to a 100 ml volumetric flask, and kept in oven at 120 °C for 24 h. After cooling the solution at room temperature, the 0.25% FA in water was added up to 100 ml as diluent. The chromatogram of the thermal degradation study is shown in Fig. 3D.

4.4. System suitability

The performance parameters of the developed UPLC system method were determined by analyzing standard working solution (400 ppm). Analytical parameters such as capacity factor (*k*) and number of theoretical plates (*N*) were calculated against the availability of ACV peak only. In addition, system repeatability was determined by calculating the relative standard deviation (% RSD) of the peak areas of six consecutive injections of working standard solution (400 ppm). From the overall analysis, the variation (% RSD) in the peak area (0.47%), capacity factor (*k* = 1.66) and number of theoretical plates (*N* = 5069.4) results shows the acceptable performance of the systems (Table 3).

4.4.1. Bioanalytical method validation

4.4.1.1. Linearity and range. The peak area response of ACV was linear over the concentration range between 1 and 400 ppm (Fig. 4). The results of linear regression give the following mean equation:

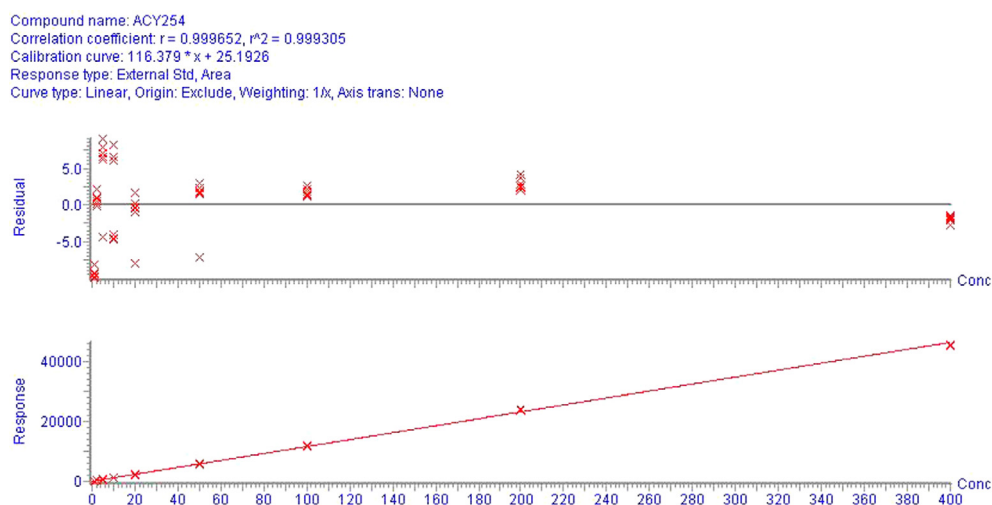


Figure 4 UPLC calibration curve of ACV in water containing 0.25% formic acid (FA).

Table 1 UPLC data of intra-day back-calculated ACV concentrations of the calibration standards in water containing 0.25% formic acid.

Nominal conc. (ppm)	Back calculated concentrations (ppm)						Mean	SD	Precision %	Accuracy %
	1st	2nd	3rd	4th	5th	6th				
1	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0	0	90
2	2.1	2.1	2	2	2.1	2	2.05	0.055	2.67	102.50
5	5.3	5.4	5.3	5.2	5.4	5.3	5.32	0.052	0.97	106.33
10	10.6	10.4	10.2	10.4	10.4	10.5	10.42	0.160	1.54	104.17
20	20	19.6	19.5	18	18	18.1	18.87	0.845	4.49	94.33
50	49.9	46.1	49.9	49.8	49.9	50.4	49.33	1.604	3.25	98.67
100	99.8	99.8	99.3	98.4	100.3	99.5	99.52	0.383	0.38	99.52
200	200.4	200.3	202.3	201.3	202.8	202.1	201.53	1.071	0.53	100.77
400	403.6	400	399.3	399.6	398.1	399.8	400.07	1.876	0.47	100.02

$$y = 113.906(\pm 1.30946)x + 21.62037(\pm 2.119667)$$

where y denotes the peak area of the analyte and x denotes the concentration of the analyte. These results show excellent linearity over the interval studied with correlation coefficient (r) = 0.9993 (± 0.007272) (Mulholland and Hibbert, 1997).

4.4.1.2. Accuracy recovery. The accuracy was calculated as the % of drug recovered after analysis relative to the corresponding nominal concentrations. The intra-day (Table 1) accuracy (mean recoveries, mean \pm SD) was found between 90% and 106.33% and the inter-day (Table 2) accuracy (mean recoveries, mean \pm SD) was found between 96.09% and 107.04%. The results from the drug recovery suggest that the accuracy of the assay method has been well matched with the acceptance criteria of FDA guidelines (US Dept. of Health and Human Services, 2001).

4.4.1.3. Precision. The results of intra-day and inter-day precision are presented in Table 1 and Table 2, respectively. The developed method was found to be precise as the intra-day standard deviation (SD) values (Table 1) of six replicate analyses were within the range of 0–1.87 ppm. In the analytical range of 1–400 ppm (conc.), the coefficient of variation (CV; precision) was only ranging from 0% to 4.49%. Moreover, the inter-day (Table 1) SD values of six replicate determinations in three consecutive days were between 0.14 and 2.84 ppm, whereas the CV being in the range of 0.47%–6.17%. These low values of both SD and CV during the intra-day and inter-day analysis indicate perfectly the precision of the current method (Karnes and March, 1993).

4.4.1.4. Specificity. The specificity of the developed UPLC method for ACV was investigated in order to obtain an indication of the possible interferences from the degradation product(s) if present. The result in Fig. 2D shows that there was no degradation products present in the ACV sample. It seems that ACV compound can be recovered completely from the lipid formulation (Fig. 2D). Therefore, the R value in this assay can be calculated based on the availability of ACV peak only (20). In addition, there were no significant interfering peaks present in randomly selected drug free lipid formulation (QC_{zero}) samples at ACV retention time (Fig. 2C), which suggests that ACV compound can be analyzed predominantly from lipid based formulations.

Table 2 UPLC data of inter-day accuracy (recovery) and precision of ACV QC samples.

Day of analysis	QC1	QC2	QC3	QC4
	1.48 ppm	64.48 ppm	287.76 ppm	377.12 ppm
1st day	1.4	66.5	289.8	377.8
	1.3	66.9	289.4	377.6
	1.3	66.6	291	373.1
	1.3	66.6	290.2	376.1
	1.3	60.8	290.4	373.8
2nd day	1.3	66.7	289.4	373.9
	1.4	69.7	290.3	376.5
	1.5	69.7	290.9	375.1
	1.4	70.2	287.9	374.7
	1.4	69.8	289.3	376.1
3rd day	1.5	69.7	290.2	376.2
	1.5	70.1	289.3	374.5
	1.5	71.4	292.6	376.6
	1.5	71.5	290.3	376.4
	1.5	71.8	290.4	376.1
Mean	1.42	69.02	290.43	375.84
SD	0.09	2.84	1.36	1.48
Precision	6.17	4.12	0.47	0.39
Accuracy	96.09	107.04	100.93	99.66

4.4.1.5. Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ). The LLOQ in the assay was 1 ppm which was estimated to be the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision for the ACV analyte, and with S/N ratio of 72.12. On the other hand, LOD was 0.1 ppm with S/N ratio of 14.15.

4.5. Application

Within the scope of the current project, the developed UPLC method has been proposed for the quantification of ACV compound in the studies of equilibrium solubility, dynamic dispersion and dissolution profiles of lipid-based formulations (Mohsin, 2012). A dispersion profile is shown in Fig. 5 as an example of the method application. The data represented an immediate release of ACV from a self-emulsifying lipid-based formulation in aqueous media (Mohsin and Alanazi, 2012).

Table 3 Systems suitability parameters for Acyclovir (ACV), $n = 6$.

System suitability parameter	ACV
Standard working solution	400 ppm
Retention time	0.89 min
% RSD	0.47%
Capacity factor (k^*)	1.66
Theoretical plate number (N^*)	5069.4

Note: *The chromatographic conditions used were: Acquity® UPLC BEH C_{18} column (2.1×50 mm, $1.7 \mu\text{m}$) kept at 50°C , mobile phase was water with 0.25% formic acid (FA) delivered at a flow rate of 0.5 ml/min, a Photodiode Array (PDA) $\text{e}\lambda$ detector at wavelength of 254 nm.

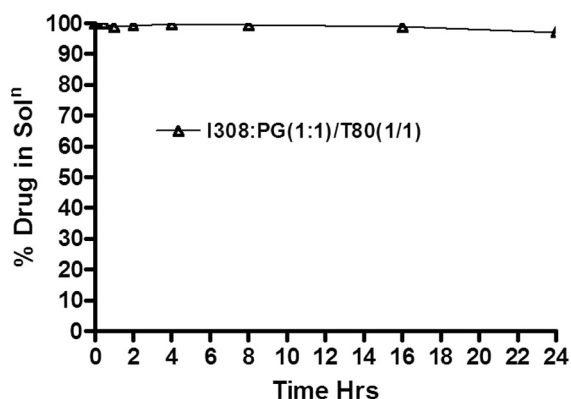


Figure 5 The dispersion (immediate release profile) of ACV from a self-emulsifying lipid-based formulation in aqueous medium. Lipid formulation represents I308: PG (1:1)/T80 (1/1). Data are expressed as mean \pm SD, $n = 3$.

The lipid formulation consisted mixture of medium chain mono-glycerides (I308), water-soluble surfactant (T80) and cosolvent (Propylene glycol) with 5 mg of ACV solubilized in them. From the results it can be stated that the current method quantified more than 98% ACV, which was released immediately after dispersion and stayed in solution during 24 h time period.

5. Conclusion

The developed UPLC analytical method provides an eco-friendly, reliable, reproducible and specific assay for ACV in pure and pharmaceutical formulations. The described method is sensitive enough to detect as low as 0.1 ppm and exclusively offers a rapid determination of ACV (peak at 0.89 min within 1.2 min run time). No significant interferences were caused by the formulation excipients, diluents and or degradation products.

The validation method allows quantification of ACV in pure and pharmaceutical formulations particularly lipid based formulations in the range between 1 and 400 ppm. Compared to previously reported methods, the present assay method assessed extensive validation parameters as per FDA guidelines. The method has shown acceptable precision, accuracy and adequate sensitivity and demands to be in use for further studies.

The established method satisfies the system suitability criteria, peak integrity, and resolution of the drug peak. The overall results clearly indicate that the current method is attractive due to the good selectivity for quantitative determination of ACV in lipid-based formulation and also suitable for stability measurements.

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